

## In the United States Patent And Trademark Office

In re Patent Application of:  
**Farooqui, F. *et al.***

Serial No.: 10/032,592

Filed: October 24, 2001

For: **Efficient Synthesis of Protein-  
Oligonucleotide Conjugates**

Examiner: Ceperley, M.

Art Unit: 1641

Atty. Dkt.: 2065-181  
(3501.143)

### Declaration Pursuant to 37 C.F.R. § 1.131

Honorable Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

1. We, Firdous Farooqui and M. P. Reddy, are the inventors of the above-identified patent application.
2. We have been advised that the claims of the present application have been rejected as anticipated (pursuant to 35 U.S.C. § 102(e)) or as obvious (pursuant to 35 U.S.C. §§ 102(e)/103(a)) in light of U.S. Patent Publication US 2003/0045694 A1 (Chait *et al.*), taken alone or in combination with U.S. Patent No. 5,648,213 (Reddy *et al.*).
3. Prior to the August 13, 2001, filing date of Chait *et al.*, we conceived of the present invention, and reduced it to practice in the United States. As evidence thereof, we have enclosed true copies of pages of the laboratory notebook of Dr. Firdous Farooqui (**Exhibit A**), as well as a true copy of an "Invention Disclosure" (**Exhibit B**).<sup>2</sup> The documents comprising **Exhibits A** and **B** were prepared and completed by us, or under our direction and supervision, in the United States prior to August 13, 2001. The experiments referred to in these Exhibits were conducted and completed in the United States prior to August 13, 2001.

<sup>2</sup>

The dates and names of witnesses have been redacted from the documents.

Declaration Pursuant to 37 C.F.R. §1.131

In Re: Appln of Farooqui, F. *et al.*

Serial No. 10/032,592

Page 2

- A. **Exhibit A**, pages 1-2 describes the synthesis of an oligonucleotide of defined sequence, its purification, 3' amination conjugation with an anti-IL-8 antibody. **Exhibit A**, page 3 describes the purification of the antibody-oligonucleotide conjugate. **Exhibit A**, pages 4-5 describe the use of the conjugate molecule to assay IL-8.
- B. **Exhibit B** describes embodiments of the present invention, including a preferred reaction scheme, and a photocopy of the results of an assay of IL-8 that was actually conducted in the United States prior to April 13, 2001, using the antibody-oligonucleotide conjugate.
- 4 We each hereby declare that all statements made herein of our own respective knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

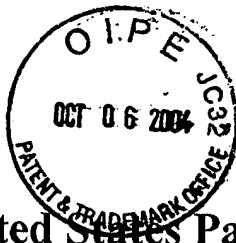
Respectfully Submitted,

Oct 04, 2004  
Date

Oct 04, 2004  
Date

Firdous Farooqui  
Firdous Farooqui

M. P. Reddy  
M. P. Reddy



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### Exhibit A

1LB - oligo conjugate

oligo- NH<sub>2</sub> <sup>3'</sup> <sup>5'</sup> CGC TAC TTC GAC AGC ATC GGC AGT.  
 CTG TAT B' 3' NH<sub>2</sub>

40 ods G-25 purified (oligo-2)

40 ods - 3' amino oligo + 4.6 mg SMCC in 500 µl of 0.1 M HEPES buffer pH 8.2  
 Incubate at rt for 1 hr. Purify on G-25  
 superfine DNA grade 1.5 x 50 cm column  
 using H<sub>2</sub>O.

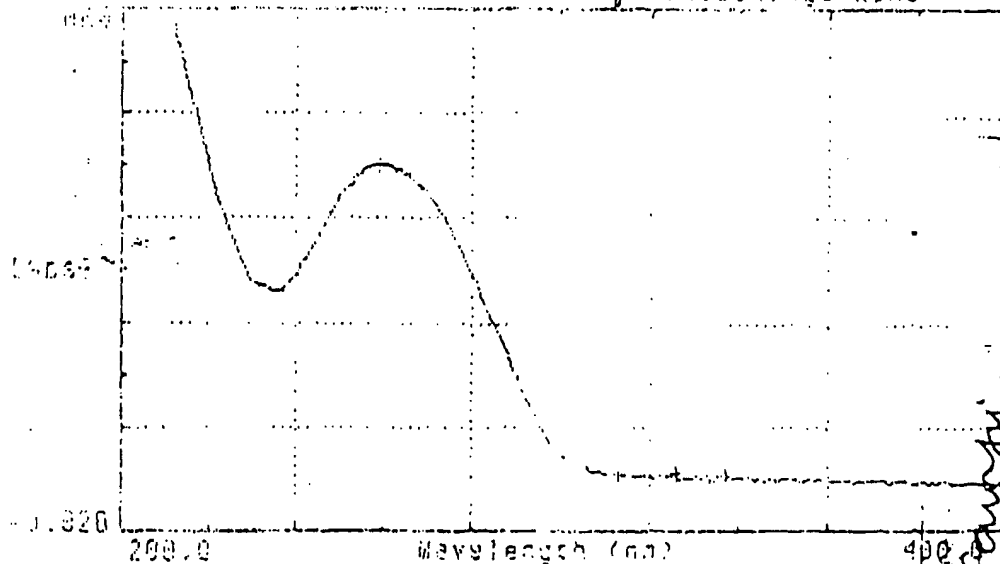
1 min fraction collector

fr. #	260 nm	
11	2.5	} mixed 11-19
12	2.5	
13	2.5	
14	1.5	
15	0.4976	

100 µl / 900 µl SMCC Peps  
 Total volume = 10 mL

SMCC oligo

259.0	0.0045	DA
260.0	0.0044	DA
262.0	0.0033	DA
312.0	0.0091	DA
326.0	0.0081	DA
334.0	0.0087	DA
392.0	0.0055	DA



100/900

Total = 39.45 ods

Farooqui  
 Witnessed and Understood  
 Date

α IL8 antibody R & D sample

2.5 mg dissolved using NAP-25 with 1X PBS  
 concentrated using centricon-30

IT 2 mg/mL  
 $138 \times 30 = 4140$   
 $100,000 \quad 4140$   
 $2.5 \quad 0.07 \text{ mg}$

α IL8 Ab in ~ 600 μL of 1X  
 + 19 μL IT solution &  
 ~ 20 μL

5 mg / 100 μL  
 0.7 mg 19 μL  
 Incubate at rt for 2h

Passed through G-25, 1X PBS 5mM EDTA

1 min fraction

fr. #	280 nm	
10	0.0017	
11	0.0017	
12	0.1628	mixed ~ 8.5 mL
13	0.5002	
14	0.0251	
15	0.0108	

Total volume = 19 mL SMCC dlyo

Add 3M salt = 3.33 gms + AB-1

2 mM EDTA = 380 μL of 100 mM EDTA

10X PBS = 1 mL

Put 2.3 mL in centricon 30 & centrifuge  
 and centrifuge at 6000 rpm for 2 hrs  
 22°C 1 hr at 6000 rpm for another  
 12°C 3 hrs & leave over 3 nights.

NACL  
 MW = 58.44  
 175.32 gms  
 1 mL  
 3M

Work Performed by: F. Farooqui  
 Witnessed and Understood: Date

P-100 column of 128 AB conjugate  
 from page (106-107) pumped

P100 column (98-180  $\mu$ ) 1.5 x 25 cm column  
 0.1 M Tris 5 mM EDTA pH 7.4 3 run fractions

f. #	260 nm	280 nm	260/280	base line
5	0.0411	0.0466		.03
6	0.0111	0.0205		.01
7	0.0423	0.0971		.02
8	0.2817	0.2675	1.053	.02
9	0.6926	0.6674	1.037	.02
10	0.5025	0.4621	1.0874	.03
11	0.3188	0.2347	1.3583	.01
12	0.3750	0.2311	1.6226	.01
13	0.5144	0.3269	1.5736	.02
14	0.6644	0.3859	1.7216	.01
15	1.2437	0.7036	1.7673	.01

Mix fractions 8-11 and pass through DEAE  
 1 run 3 mL DEAE gel, wash 10 mL 0.1 M Tris pH 8.6  
 5 mM EDTA then load sample 4x

f. #	260 nm	280 nm	260/280	base line
1 unloading sample	0.2801	0.2828	0.9904	.05
2 wash	0.1064	0.1091		.002
3 wash	0.0397	0.0363		
0.1 M salt 4	0.6357	0.6260		2 mL .006
0.1 M salt 5	0.0569	0.0493		2 mL .02
0.25 " 6 A	0.1491	0.1401	1.064	2 mL .002 0.2802
0.25 " 7 B	0.1262	0.1210	1.007	2 mL .01 0.2420
0.5 " 8 C	0.2887	0.2356	1.226	2 mL .009 0.4712
0.5 " 9 D	0.1943	0.1614	1.204	2 mL .01 0.322
1 10	0.0923	0.0819	1.1339	2 mL .01

Sample 4, 5, 6, 7, 8, 9, 10 DB 0-15 10 10 10

A+B mixed run 8% PAGE  
 C+D mixed run 8% PAGE

Total 1.25 = 0.651

LB assay using conjugate from page 117 118 - whole cell conjugate

Step I 40  $\mu$ l of reagent + 40  $\mu$ l of 0.5 salt in casein + 320  $\mu$ l of casein buffer

PP 117: Fr # 1 2 3 4  
 A B C D

Add 50  $\mu$ l in each well, shake at 37°C for 1 hr  
 wash 3x 5 min each, wash buffer from 0.5 M salt

Step II 148 1  $\mu$ l  $\rightarrow$  1 mL 10 ng/mL (100  $\mu$ l of 5 M salt + 900  $\mu$ l of casein buffer to make 0.5 M salt)  
 25  $\mu$ l  $\rightarrow$  1 mL 250 pg/mL  
 400  $\mu$ l  $\rightarrow$  1 mL 100 pg/mL  
 250  $\mu$ l  $\rightarrow$  1 mL 25 pg/mL

Add 50  $\mu$ l & shake at 37°C for 1 hr  
 wash 3x, 5 min each.

Step III PB-Erwin 1:100  
 10:1000  
 20:2000

1 1/2 hr at 37°C, wash 3x 5 min each

Step IV SAV-AP SAV-PBAL } 50 min at 37°C  
 1:500 1:100  
 2:1000 10:1000

Substrate

1:20

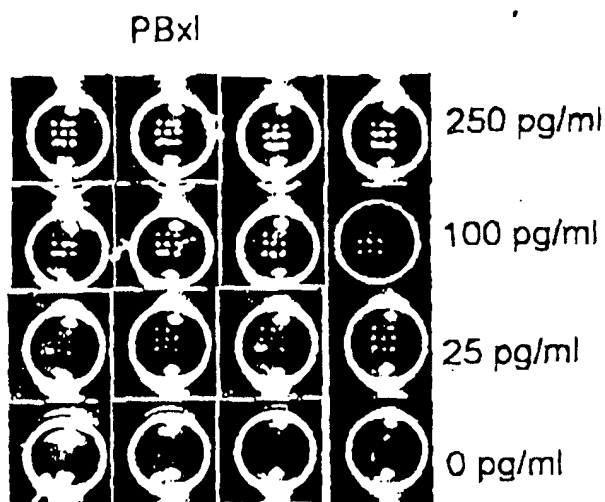
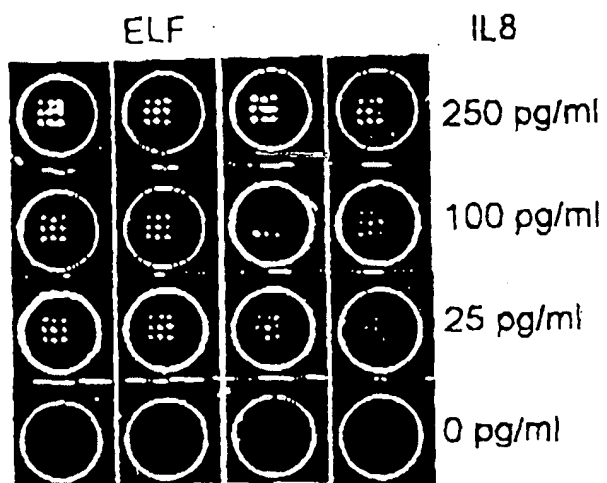
10:200

40:800

30 min at rt No shaking

Wash & take picture.

Fraser



Work Performed by: *F. Farooqui*  
 Witnessed and Understood: *[Signature]*  
 Date: *[Blank]*  
 Date: *[Blank]*

Mixed fractions (A+B from page 117) IL8 - whole Ab  
 (C+D) conjugate  
 run on gel 10% denaturing page by Dean.  
 Conclusion: IL8 - Ab conjugate performs well  
 in assay





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**Exhibit B**

## INVENTION DISCLOSURE

1. **Title of Invention:** Efficient synthesis of protein-oligonucleotide conjugates.
2. **Product line in which invention will be used:** Immediate use in Protein array or A<sup>2</sup> plate project. Potential use in bead based flow project. This new chemistry may be used to manufacture protein-oligonucleotide conjugates in high yields.
3. **Summary of the Invention:** Oligonucleotide-antibody conjugates are useful reagents in immunoassays<sup>1</sup>. The covalent attachment of an oligonucleotide moiety to antibody provides a specific recognition site for a complementary nucleic acid and can subsequently be used as a molecular handle for selective immobilization and construction purposes. In a multiplexed immunoassay approach, oligonucleotide arrays printed on A<sup>2</sup> plate serve as a universal capture reagent. The complimentary oligonucleotides are conjugated to the corresponding antibodies and the assay is performed. The antibody-conjugated probe prepared by the method disclosed here gave high sensitivity.

Synthesis of oligonucleotide-antibody conjugate is accomplished in four steps:

1) Synthesis of 3'-amino oligonucleotide 2) 3'-amino oligonucleotide is activated with a hetero bifunctional linker, sulfo SMCC 3) antibodies are thiolated using iminothiolane (Traut's reagent) 4) The activated oligonucleotide and thiolated antibodies are then mixed to facilitate coupling of oligonucleotide to antibody (see Figure 1). Antibody-oligonucleotide conjugates are characterized by PAGE and tested for their biological activity by performing immunoassay (see Figure 2). This approach is simpler or higher yielding as compared to the procedures known in literature: thiol group is introduced at the 5' or 3' end of oligonucleotide

Inventors Name:		Date
Firdous Farooqui	<i>F. Farooqui</i>	
M. Parameswara Reddy	<i>M. Parameswara Reddy</i>	
Witnessed and Understood		Date
Witnessed and Understood		Date

antibody was purified on G-25 column, 1.5 cm x 50 cm, using 1 x PBS, 5 mM EDTA as an eluent.

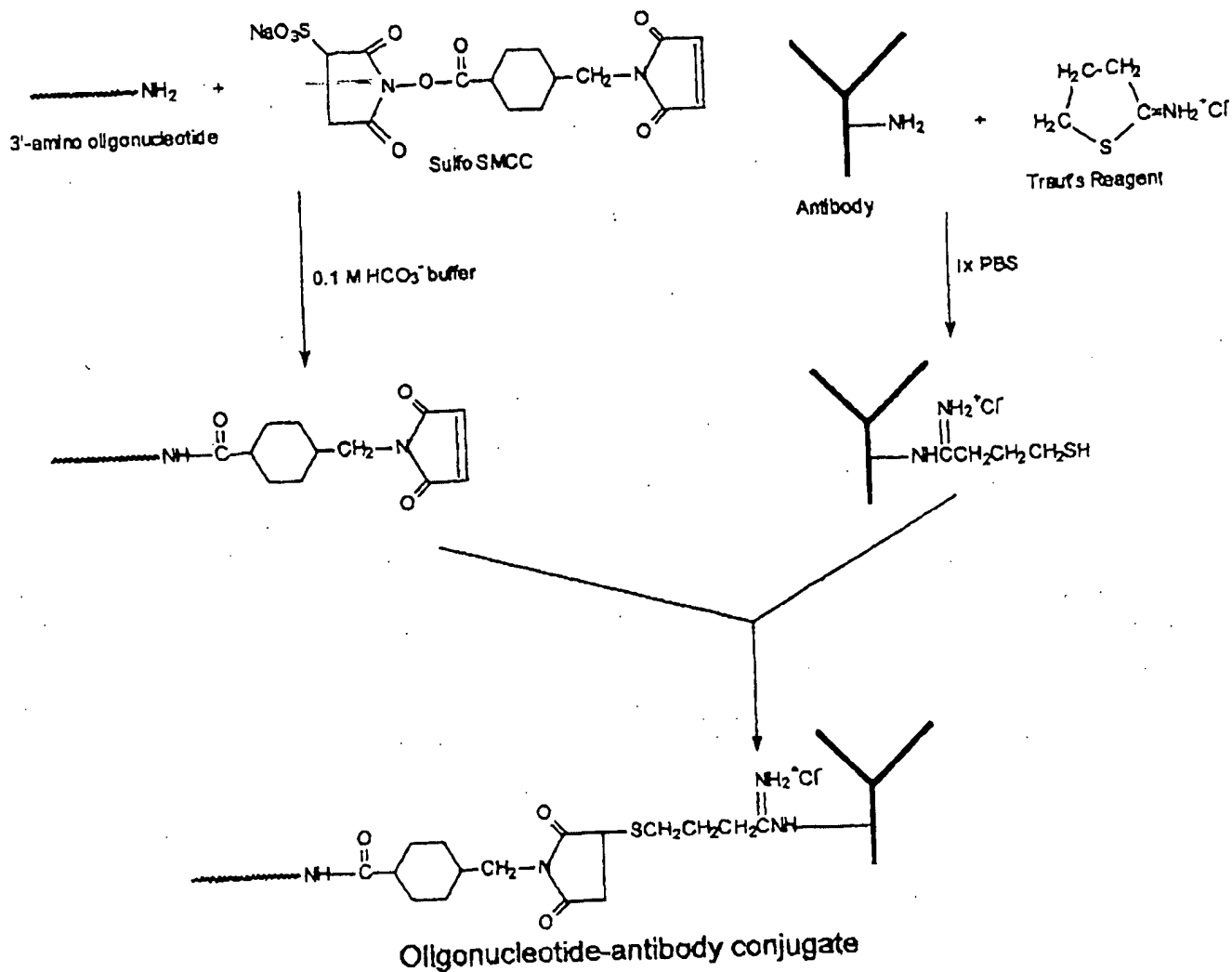
4. DNA oligonucleotide-Ab conjugation: Activated antibody was mixed with sulfo SMCC oligonucleotide, the final conc. was 1 x PBS, 3 M NaCl, 2 mM EDTA. The resulting mixture was concentrated using centricon-3 for 3 hours and left to react at 12°C for 3 nights.
5. Purification of Oligonucleotide antibody conjugates: The Antibody-oligonucleotide conjugate was purified on size exclusion P-100 column, 1.5 cm x 20 cm, using 0.1 M tris buffer pH 7.2, 5mM EDTA as an eluent. The first band collected which had 260/280 ratio of 1.0- 1.2 were mixed and purified on DEAE column with salt gradient from 0.5 M NaCl to 1 M NaCl. The yield of the conjugate was 25-35%. The conjugate was characterized on 8% PAGE and stained with stains all.
6. Immunoassay Protocol: The complementary oligonucleotide was printed on A<sup>2</sup> plate 3 x 3 array. The 140 ng of Antibody-oligonucleotide in casein buffer with 0.5 M salt was added to the well and incubated for 1hr at 37°C. Wash the plate 3x, added antigen in different conc. ranging from 1000 to 1 pg/ml in 50 ul of volume, wash 3x, added secondary antibody conjugate with biotin react for 1 hour at 37°C, wash 3x, and added SAV-PBXsl react for 1hour wash 3x and imaged the plate under CCD camera. The sensitivity of the assay was found to be 1 pg/ml with IL8 antigens (see Figure 2).

Inventors Name:	Date
Firdous Farooqui <i>F. Farooqui</i>	
M. Parameswara Reddy <i>M. Parameswara Reddy</i>	
Witnessed and Understr-	Date
Witnessed and Understood	Date



**Figure 1**

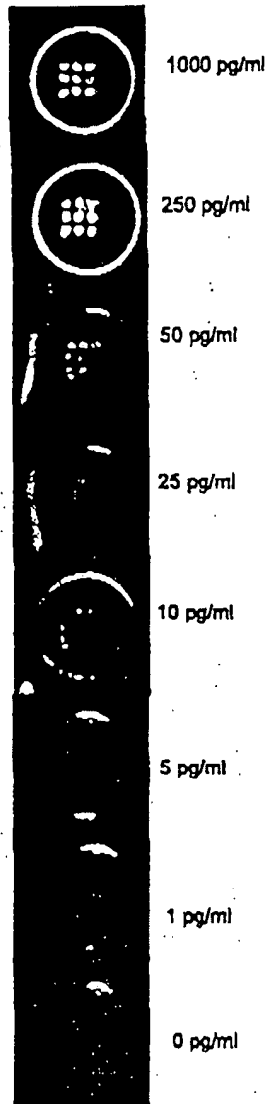
**Synthesis of Oligonucleotide-Antibody Conjugate**



Inventors Name:		Date
Firdous Farooqui	<i>F. Farooqui</i>	
M. Parameswara Reddy	<i>M. Parameswara Reddy</i>	
Witnessed and Understood		Date
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Figure 2

IL 8 assay on A<sup>2</sup> Plate



Inventors Name:	Date
Firdous Farooqui <i>F. Farooqui</i>	
M. Parameswara Reddy <i>M. Parameswara Reddy</i>	
Witnessed and Understood	Date
Witnessed and Understood	Date

**Claims:** Synthesis of oligonucleotide-antibody conjugates where oligonucleotide is activated with a hetero bifunctional linker, antibody is thiolated and mixed to form a conjugate.

**References:**

1. M. P. Reddy, J. C. Sternberg; US patent # 5,648,213.
2. Yarmush M. L. et al; Bioconjugate chemistry 8 (6) 935-940, 1997.
3. Hendrickson R. E. et al Nucleic Acid Research 23 (3), 522-529, 1994.
4. DNA Probes, George H. Keller, Mark M. Manak, MacMillan Publishers Ltd, 1989.

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